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RESEARCH ARTICLE

Syk is indispensable for CpG-induced activation and differentiation of human B cells

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Abstract B cells are efficiently activated by CpG oligodeoxynucleotides (ODNs) to produce pro-inflammatory cytokines and antibody (Ab). Here, we describe a so far unidentified, spleen tyrosine kinase (Syk)-dependent pathway, which is indispensable for CpG-induced human B cell activation. We show that triggering of B cells by CpG results in Syk and src kinase phosphorylation, proliferation, as well as cytokine and Ab production independent of the BCR. Notably, all these functions are abrogated when Syk is inhibited. We demonstrate that CpG-induced Syk activation originates from the cell surface in a TLR9-dependent manner. While inhibition of Syk does not influence the uptake of CpG ODNs, activation of the kinase is a prerequisite for the delivery of CpG into TLR9-containing endolysosomes and for the CpG-induced up-regulation of TLR9 expression. Our results reveal an alternative, Syk-dependent pathway of CpG-induced B cell stimulation, which is initiated at the plasma membrane and seems to be an upstream requirement for endosomal TLR9-driven B cell proliferation and differentiation.

Keywords B cells · TLR9 · Syk · Cell activation · Signal transduction

Abbreviations

ODN	Oligodeoxynucleotide
TLR	Toll-like receptor
MyD88	Myeloid differentiating factor 88
Syk	Spleen tyrosine kinase
BCR	B cell receptor
MAPK	Mitogen-activated protein kinase
LAMP-1	Lysosome-associated membrane protein-1

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Introduction

To discriminate between self and non-self structures, the immune system has evolved a series of pattern recognition receptors to identify pathogens and initiate the host's immune response. Members of the TLR family function in this fashion and are able to activate both the innate and adaptive arms of the immune response [1]. Human B cells express a diverse repertoire of TLRs, in which TLR1, TLR7, TLR9 and TLR10 are predominant [2].

TLR9 has been reported to be the major receptor for unmethylated CpG ODNs found within viral and bacterial but not in human DNA [3]. Bacterial DNA is efficiently mimicked by synthetic CpG ODNs, of which the 23mer nuclease-resistant phosphorothioate ODN 2006 seems to be the most potent agonist for human B cell stimulation [4, 5]. TLR9 is expressed both at the cell surface and intracellularly in human B cells [5–7]. Activation of B lymphocytes by CpG results in the up-regulation of activation markers, as well as proliferation, cytokine and Ab production [5, 8–10]. The signal pathway mediated by the engagement of TLR9 is known to involve MyD88-induced NF-κB activation [11–13]. TLR9 and the BCR synergize to induce class switch recombination in both T cell-dependent and T cell-independent responses at the level of mitogen-activated protein kinases (MAPKs) [14]. This strong co-

operation results in enhanced proliferation and differentiation of B cells [15], which may contribute to the development and pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) [16]. Coupling B cell receptor (BCR) stimulation to TLR9 expression and function endows the human system with a highly specific immune response, since it allows focusing of innate signals only on Ag-stimulated B cells.

It also has to be considered that CpG may bind to sensors other than TLR9, thus the CpG-induced pathway is not necessarily identical to the TLR9-mediated activation route. Several studies have shown that human B cells can be stimulated even without the uptake of phosphorothioate ODNs, by binding to cell surface structures [17, 18], including scavenger receptors (DEC-205 and SR-B1) [19, 20], complement receptor type 2 [21], surface Igs [22] and TLR9 itself [23].

Syk is a 72-kDa non-receptor tyrosine kinase which, among other important biological functions, has a crucial role in immunoreceptor signaling [24]. Upon Ag binding by the BCR, two tyrosines within the immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα (CD79A) and Igβ (CD79B) are rapidly phosphorylated by src-family kinases leading to Syk activation [25]. Deletion of Syk results in a partial block in B cell development at the pre B cell stage, preventing formation of mature B lymphocytes in the periphery [26, 27]. Results published recently by Schweighoffer et al. [28] confirm that Syk is fundamental in maintaining tonic survival signals induced by the BCR and B cell activating factor receptor. Furthermore, Syk has been proven to be a crucial player in signal integration during human B cell activation induced by CD40 and the BCR [29].

Although the important role of Syk in immunoreceptor-mediated signaling pathways has been established earlier and its role in TLR-associated innate immunity has raised attention, their possible interaction has not been studied yet. Jabara et al. [30] showed that after ligation of TLR9 by CpG ODNs, DOCK8 links TLR9 to a src kinase-Syk cascade which is essential for TLR9-driven B cell proliferation and differentiation. Furthermore, studies using the human monocytic THP-1 cell line and mouse splenic B cells proved that CpG ODN induces phosphorylation of src-family kinases in a TLR9-independent manner, resulting in cell adhesion and motility. This signaling pathway intersects the conventional TLR9-MyD88 pathway by promoting the tyrosine phosphorylation of TLR9 and the recruitment of Syk to this receptor [31]. It was also established that Syk-mediated BCR triggering is a prerequisite for optimal induction of TLR9-induced signaling in human B cells [32]. However, it remains unclear whether Syk can directly be activated in the course of the TLR9 signaling pathway or not.

Here, we identify a so far not described, Syk-dependent pathway which is indispensable for the CpG-induced activation of human B lymphocytes. We show that stimulation of B cells by CpG ODN results in phosphorylation of Syk as well as proliferation, cytokine and Ab production. Importantly, the inhibition of the tyrosine kinase abrogates all these basic CpG-mediated B cell functions. Our results reveal an alternative pathway of B cell stimulation, which is initiated by cell surface-bound CpG and involves Syk and src kinases.

Materials and methods

Reagents and antibodies

For B cell stimulation the following reagents were used: CpG 2006, non-activating control GpC 2006, 5′ biotinylated CpG 2006 (synthesized by Sigma-Aldrich) and $F(ab')_2$ fragment of goat anti-human IgG/A/M (Jackson ImmunoResearch). Syk inhibitor IV (iSyk IV, BAY61-3606), Syk inhibitor I (iSyk II) and Src kinase inhibitor were purchased from SantaCruz Biotechnology. siRNAs were purchased from SantaCruz Biotechnology. Dimethylsulfoxide (DMSO) was from Sigma-Aldrich. MyD88 inhibitor was purchased from Invivogen. H^3-thymidine and CFSE were from American Radiolabeled Chemicals and Invitrogen (Life Technologies), respectively. FlowCytomix Kits for cytokine measurement assays were from eBioscience (human IL-6, IL-10 and TNF-α Simplex Kit). Recombinant cytokines (IL-2 and IL-10) were purchased from Immunotools. ELISPOT assays were carried out with the following Abs: anti-human IgM or IgG (BD Pharmingen) and HRP-conjugated anti-human IgM purchased from DAKO or anti-human IgG obtained from BD Pharmingen.

Abs for flow cytometry were either biotinylated or conjugated with FITC, PE, APC or PerCP-Cy5.5 and were as follows: anti-CD38 (clone HIT2, Immunotools), anti-CD27 (clone CLB-72/1, Invitrogen), anti-CD19 (clone LT19, Immunotools), anti-TLR9 (clone: 26C593 or clone: H-100, SantaCruz Biotechnology), anti-Syk (SantaCruz Biotechnology), anti-CD86 [clone 2331 (FUN-1), BD Pharmingen], anti-CD80 (clone L307.4, BD Pharmingen), anti-CD40 (clone HI40a, Immunotools), streptavidin-PerCP-Cy5.5 (BD Pharmingen). As isotype control, unconjugated mouse IgG1 (SantaCruz Biotehnology) and isotype-matched FITC-, PE- and APC-conjugated mouse Igs (Immunotools) were used to assess nonspecific staining. Fluorescent labeled CpG-Alexa Fluor 488 was synthesized by Integrated DNA technologies. Lysosome-associated membrane protein 1 (LAMP1) and TLR9 (clone: 26C593) antibodies used for confocal microscopy were purchased from Sigma-Aldrich and SantaCruz Biotechnology,

respectively. Alexa Fluor-conjugated secondary antibodies were from Invitrogen. If not mentioned otherwise, Abs used for Western blot analyses were purchased from Cell Signaling Technology and were the followings: anti-TLR9 (clone: 26C593, SantaCruz Biotechnology), anti-phospho-Syk (pSyk, sc-293118, SantaCruz Biotehnology), anti-CD79A (#5173) anti-phospho-p38 (pp38, #9211), anti-phospho-p44/42 MAPK (pERK1/2, #4370), anti-phospho-Lyn (pLyn, clone EP503Y), SHP-2 (clone 79/PTP1D/SHP2, BD Transduction Laboratories), β-actin (#4970). As secondary Ab, HRP-conjugated rabbit anti-mouse IgG (sc-358914, SantaCruz Biotechnology) or HRP-conjugated goat anti-rabbit Igs (P0448, Dako) were used. Cell extracts were prepared using lysis buffer containing 50 mM HEPES (pH 7.4), 1 % Triton X-100, 100 mM sodium fluoride, 10 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 % glycerol, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 5 μg/ml leupeptin and 2 mM PMSF (the inhibitors were all purchased from Sigma-Aldrich).

Cell preparation

Tonsils from children undergoing routine tonsillectomy were obtained from the Saint Istvan and Saint Laszlo Hospital in Budapest, Hungary, after obtaining written informed consent according to the Declaration of Helsinki. Tonsillar mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (GE Healthcare). After rosetting with 2-aminoethylisothiouronium bromide-treated sheep RBC, B cells were isolated by centrifugation over Ficoll-Hypaque solution. Separated B cells were further fractionated into low- and high-density populations on a Percoll (Sigma-Aldrich) gradient. $CD19^+$ B cell purity was higher than 95 % in each case. High-density ("resting") tonsillar B cells were cultured at 2×10^5 cells/well in 100 μl RPMI 1640 medium (Sigma-Aldrich) supplemented with 10 % FCS (Gibco) and 50 μg/ml gentamycin in 96-well microtiter plates (Costar).

Assays for B cell functions

2×10^5 high-density B cells were stimulated with various concentrations of CpG 2006 in the presence or absence of different concentrations of kinase inhibitors (iSyk IV, iSyk II or Src kinase inhibitor I). Inhibitors were present throughout the culture period in concentrations ranging between 0.1 and 5 nM. As control, only medium, non-activating GpC 2006 or vehicle (DMSO) treated cells were used. All cultures were analyzed for cell viability by propidium iodide exclusion and results were normalized to the number of living cells. Cell proliferation was assessed either by H^3-thymidine incorporation after 48 h or with CFSE on day 3 as described earlier [33, 34]. Secreted IL-6, IL-10 and TNF-α levels were measured by the FlowCytomix technology according to the instruction of manufacturers.

To induce plasmablast differentiation, B lymphocytes were cultured in the presence of 50 ng/ml IL-2 and IL-10. After 7 days of culture, the number of $CD19^{low}CD27^{high}CD38^+$ plasmablasts was determined by flow cytometry as described earlier [33]. To measure the number of IgM and IgG Ab secreting cells, after 7 days of culture, ELISPOT assays were performed on 96-well ELISPOT plates (Millipore) coated with anti-human IgM or IgG. After washing and blocking with PBS containing 1 % FCS, the plates were incubated with 2×10^5 activated cells for 24 h at 37 °C. After washing, the assay was developed with HRP-conjugated anti-human IgM (1:1,000) or IgG (1:2,000). For visualization, 3-amino-9-ethyl-carbazole (Sigma-Aldrich) was used as chromogen.

To assess B cell activation induced by surface-bound CpG ODN, 96-well microtiter plates (Costar) were coated with 5 μg/ml neutravidin (ThermoScientific) and different concentrations of biotin-labeled CpG 2006 or PBS alone (untreated cells). After extensive washing of the plate, 2×10^5 high-density B cells/well were incubated for 48 h. Proliferation was measured by H^3-thymidine incorporation and IL-6 or IL-10 production by the FlowCytomix technology.

siRNA silencing

Transient transfection of B cells was performed using the Amaxa human B cell nucleofector kit (Lonza) according to the manufacturer's instructions. Briefly, 1×10^6 cells were resuspended in 100 μl of nucleofector solution, mixed with 1 or 2 μM of gene-specific or control non-targeting siRNAs. Nucleofection was performed using program U-015. After transfection, cells were transferred to 500 μl preheated complete medium (RPMI 1640/10 % FCS) in a 24-well plate and incubated for 72 h at 37 °C. Viability of cells was evaluated by 7-AAD staining (BD Pharmingen). Down-regulation of protein expression was monitored by flow cytometry.

Flow cytometry

Immunofluorescence measurements were performed using a FACSCalibur flow cytometer (BD Biosciences) and the FCSExpress software, version 3.0, as described earlier [33]. On the basis of forward- and side-scattering, isolated live B lymphocytes were gated and dead cells were excluded. To analyze TLR9 expression, isolated B cells were fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences). To reduce nonspecific Ab binding, cells were treated with Fc receptor blocking

reagent (Miltenyi Biotec), followed by staining with rabbit polyclonal anti-human TLR9 (clone: H-100) for 30 min. After extensive washing, cells were labeled with anti-rabbit Alexa Fluor 647. To analyze the effect of Syk inhibition on CpG-induced B cell activation, B cells were cultured as described above and stained with anti-CD80, anti-CD86 and anti-CD40 after 48 h. To measure the uptake of CpG by B lymphocytes, cells were incubated with 1 μg/ml CpG-Alexa Fluor 488 in the presence or absence of 10nM iSyk IV at 4 °C or 37 °C for the indicated times. To demonstrate the amount of intracellular CpG, data are expressed as delta mean fluorescence intensity (MFI) of CpG-FITC bound at 37 °C and at 4 °C ($MFI_{37\ °C} - MFI_{4\ °C}$).

Laser scanning confocal microscopy

CpG ODN uptake and its colocalization with TLR9 and LAMP-1 were visualized by confocal microscopy. Briefly, B cells were stained with CpG-Alexa Fluor 488 for 30 min at 37 °C in the presence or absence of 10nM iSyk IV. After fixation and permeabilization, cells were stained with mouse anti-human TLR9 (clone: 26C593) and rabbit anti-human LAMP-1 for 30 min at 4 °C. After extensive washing, cells were labeled with anti-mouse Alexa Fluor 647 and anti-rabbit Alexa Fluor 555. Analysis was carried out using an Olympus FLUOView 500 laser-scanning confocal microscope (Hamburg, Germany). Fluorescence and DIC images (1,024 × 1,024 pixels) were acquired using a 60× oil-immersion objective. Images were processed by ImageJ software (http://rsbweb.nih.gov/ij).

Western blot analyses

To assess B cell activation induced by surface-bound CpG ODN or anti-TLR9 Ab, 24-well microtiter plates (Costar) were coated with neutravidin (ThermoScientific) and different concentrations of biotin-labeled CpG 2006 or $F(ab')_2$ fragment of anti-rabbit IgG (Jackson ImmunoResearch) and rabbit anti-TLR9 Ab (clone H-100). After overnight incubation at 4 °C, 2×10^6 high-density B cells were added to the plates and stimulated for 30 min at 37 °C.

To analyze the effect of Syk inhibition on the synergistic stimuli of BCR and CpG, 2×10^6 B cells/sample were activated with 0.1 μg/ml $F(ab')_2$ fragment of goat anti-human IgG/A/M for 2 min and/or 1 μg/ml CpG for 10 min in the presence or absence of 10 nM iSyk IV.

To analyze the involvement of TLR9 in the CpG-induced phosphorylation of Syk, 5×10^5 TLR9-specific siRNA or control siRNA treated B cells/sample were activated with 2 μg/ml CpG for 20 min or left untreated.

The effect of different inhibitors on CpG-induced phosphorylation of Syk was assessed by pre-treatment of 2×10^6 B cells/sample either with 5 μM MyD88 inhibitor, 10 nM Src kinase inhibitor I or vehicle (DMSO) for 30 min at 37 °C. After incubation, cells were activated—without washing out the inhibitors—with 1 μg/ml CpG 2006 for 20 min or left untreated.

To analyze TLR9 expression after CpG stimulation, 4×10^5 high-density B cells/well were activated with 2 μg/ml CpG in the presence or absence of 5 nM iSyk IV or vehicle control. After 48 h, 10^6 living cells/activation were sorted using a BD FACSAria III cell sorter (BD Biosciences).

In each case, activated cells were extracted in 1 % Triton X-containing lysis buffer, and cell extracts were centrifuged at 15,000*g* for 15 min at 4 °C. Protein concentration was measured by Bicinchoninic Acid Kit (Sigma-Aldrich) and 50 or 100 μg protein per sample was boiled in reducing sample buffer, separated on 10 % polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad Laboratories). After blocking with 5 % BSA, blots were incubated with the relevant primary Abs overnight, at 4 °C, followed by incubation with HRP-conjugated Abs. Bound Abs were visualized by the enhanced chemiluminescence (ECL) method (Millipore) and analyzed with the ImageJ software. Relative intensity was calculated by dividing the peak densitometry values of the target protein by the peak values of the loading control.

Statistical analysis

Statistical differences were assessed by Permutation test or Student's *t* test where appropriate, using PrismSoftware, version 4.0 (GraphPad Software). Values of $p < 0.05$ were considered significant.

Results

Binding of CpG 2006 to resting human B cells induces dose- and time-dependent phosphorylation of Syk and Lyn in a BCR-independent manner

Upon recognition of CpG-rich sequences in the endosome of human B cells, TLR9 initiates a conserved signaling cascade through the recruitment of MyD88, providing a mechanism for innate immunity to regulate the adaptive immune response [35]. Our aim is to reveal how the activation and function of human B cells is influenced by the TLR-agonist CpG ODN bound to the cell surface, and whether Syk is involved in this process.

First we set out to investigate whether Syk is involved in the CpG-initiated activation of human B lymphocytes. As seen in Fig. 1a, treatment of resting tonsillar B cells with increasing amounts of CpG 2006 caused a dose-dependent phosphorylation of Syk and the membrane proximal src

kinase Lyn. We also show that the phosphorylation of these kinases is time-dependent (Fig. 1b). These results extend and strengthen earlier observations obtained using blood derived cells, showing Syk phophorylation upon CpG treatment of B cells [30].

Surface Igs are known to interact with phosphorothioate ODNs by charge–charge interactions [22]. To rule out the possibility that under our experimental conditions CpG activates the src-Syk kinase pathway by binding to the BCR, we investigated whether Igα (CD79A), one of the signaling moieties of the BCR [25] is phosphorylated upon addition of CpG. As shown in Fig. 1c, we detected no phosphorylation of the Igα upon the CpG stimulus, in contrast to the activation by anti-Ig, confirming the assumption that the ODN-induced activation of Syk in resting human B cells does not occur via cell surface BCR.

The role of Syk and Lyn in the CpG-induced activation, proliferation and cytokine production of B cells

Next, we set out to explore whether activation of Syk and Lyn is indispensable for the CpG-induced B cell functions. To this end, high-density B cells were stimulated with a suboptimal amount of CpG 2006 in the presence or absence of different concentrations of Syk and src kinase (Lyn) inhibitors, followed by assessing various functions of the cells. Since Syk relays tonic B cell survival signals and cell viability decreases in the presence of the inhibitor, dead cells were excluded from all measurements using propidium iodide and data were normalized to the number of living cells. To reduce side-effects, the optimal concentrations of the inhibitors were titrated in preliminary experiments. As seen in Fig. 2a, we found that treatment of resting B cells with nanomolar concentrations of Syk inhibitor IV resulted in a strong and dose-dependent decrease of the CpG-induced proliferation. These results were confirmed by employing another Syk inhibitor (Syk inhibitor II) as well as a src kinase inhibitor (Fig. 2a). Due to its strong effect, we used Syk inhibitor IV in further experiments.

We went on to test the effect of Syk inhibition on the TLR agonist-induced changes in the phenotype of B cells. Consistent with our results shown previously, Syk inhibitor IV blocked strongly and dose dependently the CpG-induced up-regulation of the costimulatory molecules CD80, CD86 and CD40 (Fig. 2b). Since CpG-stimulated B cells are known to produce several cytokines and chemokines [36], next we set out to investigate whether inhibition of Syk also influences the production of prominent B cell derived cytokines. We found, that the secretion of the regulatory IL-10, as well as the pro-inflammatory IL-6 and TNF-α was diminished in the presence of the kinase inhibitor (Fig. 2c).

Since small molecule inhibitors may have potential off-target effects, the role of Syk in the CpG-induced activation of B cells was further strengthened by siRNA-mediated gene silencing. As shown in Fig. 2d–e, down-regulation of Syk expression by Syk specific siRNA resulted in decreased CpG-induced proliferation, as well as IL-6 and IL-10 production of human B cells compared to the control siRNA-sample, proving the critical role for Syk in the CpG-induced proliferation, activation and cytokine production of human B lymphocytes.

Syk regulates CpG-driven B cell differentiation and antibody production

Upon activation by CpG 2006, B cells undergo isotype switching, differentiate into plasmablast and secrete Igs [4, 8, 37]. In view of our findings described above, we aimed to reveal whether plasmablast formation and antibody production are also influenced by the inhibition of Syk. As shown in Fig. 3, Syk inhibitor IV caused a dose-dependent reduction of CpG ODN-induced differentiation of high-density B cells into $CD27^{high}CD38^{+}CD19^{low}$ plasmablasts (Fig. 3a, b), and in consequence, the number of IgM and IgG secreting cells also decreased (Fig. 3c). These data suggest that Syk is strongly involved in the regulation of CpG-driven B cell differentiation and Ab production.

Inhibition of Syk prevents the synergistic activation of MAPKs induced by BCR and CpG

Stimulation of B cells via the BCR and CpG is known to initiate the synergistic activation of MAPKs and NF-κB, resulting in enhanced proliferation as well as up-regulation of costimulatory molecules and isotype switching [38, 39]. In order to find out if Syk might coordinate the convergence of these two signaling pathways, we monitored how the upstream events, namely the phosphorylation of Syk and the membrane proximal Lyn are affected by the simultaneous stimuli. As shown in Fig. 4a, the phosphorylation of both Lyn and Syk was significantly enhanced when resting tonsillar cells were activated via their BCR in the presence of CpG, highlighting that these signaling pathways converge already at the level of Lyn and Syk. Next we investigated whether inhibition of Syk has any effect on the phosphorylation of ERK, the first MAPK where the BCR- and CpG-induced signaling pathways might merge. Using Syk inhibitor IV we found that blocking the kinase prevents not only the BCR-, but also the CpG-initiated phosphorylation of ERK (Fig. 4b), underlining the indispensable role for this tyrosine kinase in the synergistic stimulatory effect of BCR and CpG.

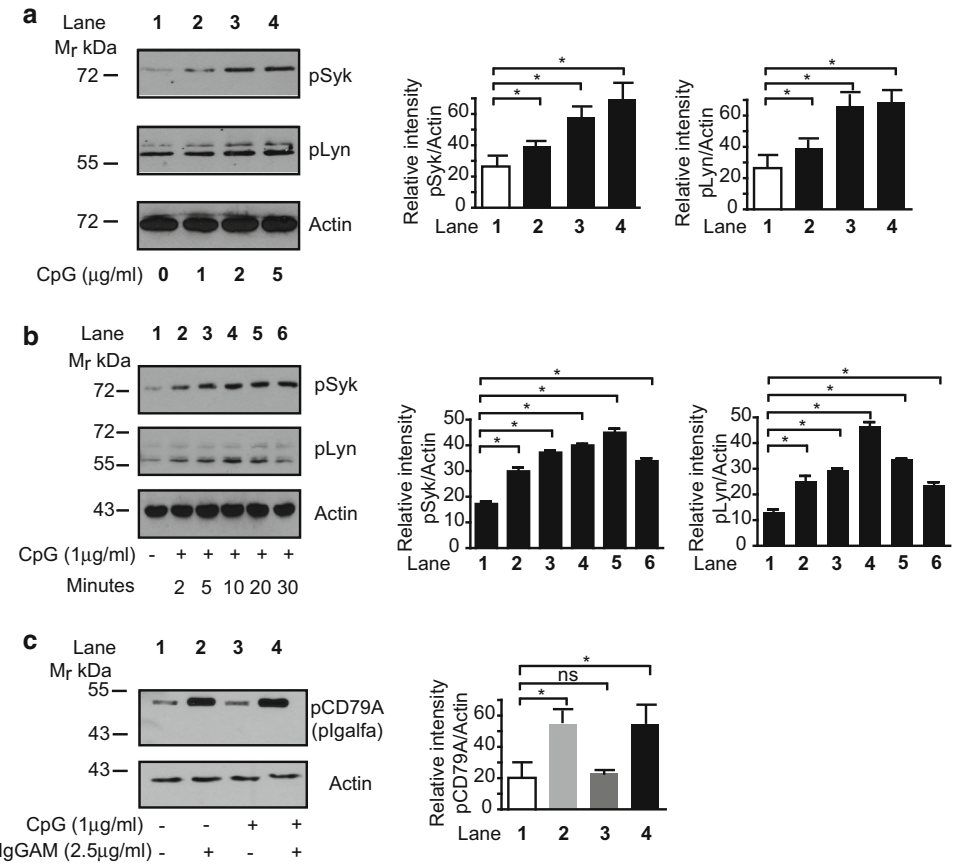

Fig. 1 Binding of CpG ODN to resting human B cells induces dose- and time-dependent phosphorylation of Syk and Lyn independent of the BCR 2×10^6 resting B cells/sample were activated with different concentrations of CpG for 30 min **a** or with 1 μg/ml CpG for different time intervals **b**. Immunoblots show results of one representative experiment of 3. *Graphs* show summarized densitometric analysis of Syk and Lyn phosphorylation as mean relative intensity ± SEM of 3 independent experiments (permutation-test, nsP > 0.05; *$P < 0.05$). **c** CpG does not induce phosphorylation of CD79A in high-density B cells. Untreated cells (*lane 1*), cells activated with 2.5 μg/ml $F(ab')_2$ fragment of goat anti-human IgG/A/M (*lane 2*), with 1 μg/ml CpG (*lane 3*) or via both stimuli simultaneously (*lane 4*). Immunoblot shows one representative experiment of 3. Graph shows summarized densitometric analysis of CD79A phosphorylation as mean relative intensity ± SEM of 2 independent experiments (permutation-test, ns $P > 0.05$; *$P < 0.05$)

Both intracellular and cell membrane TLR9 is involved in the CpG ODN-induced Syk activation

CpG-induced Lyn and Syk phosphorylation has been demonstrated to occur both in a TLR9-dependent [30] and -independent [31] manner in human B cells. This implies that the CpG-induced signaling pathway is not necessarily identical to the TLR9-mediated activation route. Indeed, it has been shown that CpG may bind to other sensors than TLR9 [19–23]. To analyze the involvement of TLR9 in the CpG-induced phosphorylation of Syk, we knocked-down TLR9 in isolated human B cells by siRNA-mediated gene silencing. As shown in Fig. 5a, down-regulation of TLR9 employing 2 μM siRNA led to an approximately 50 % decrease in Syk activation. The effect of RNA-silencing on TLR9 expression was also followed by FACS analysis of the transfected cells and showed a two-fold decrease in the case of 2 μM specific siRNA (data not shown). To further prove the involvement of TLR9 in the CpG-induced phosphorylation of Syk we moved on to test whether inhibition of MyD88, a critical adaptor protein of TLR9-induced signaling events [11] affects the CpG-driven activation of the kinase. As shown in Fig. 5b, blockade of MyD88-mediated signaling pathways in human B cells prevented the CpG-induced phosphorylation of Syk, similarly to the control, src kinase inhibitor, confirming the role of TLR9 in the kinase activation.

Activation of Syk was reported to originate both at the plasma membrane, upstream of intracellular TLR9 [31] and intracellularly, after ligation of endosomal TLR9 [30]. To test whether CpG might induce Syk phosphorylation through cell surface TLR9, first we analyzed the receptor expression in resting human tonsillar B cells. Similarly to earlier reports on blood derived B lymphocytes [7, 23] we

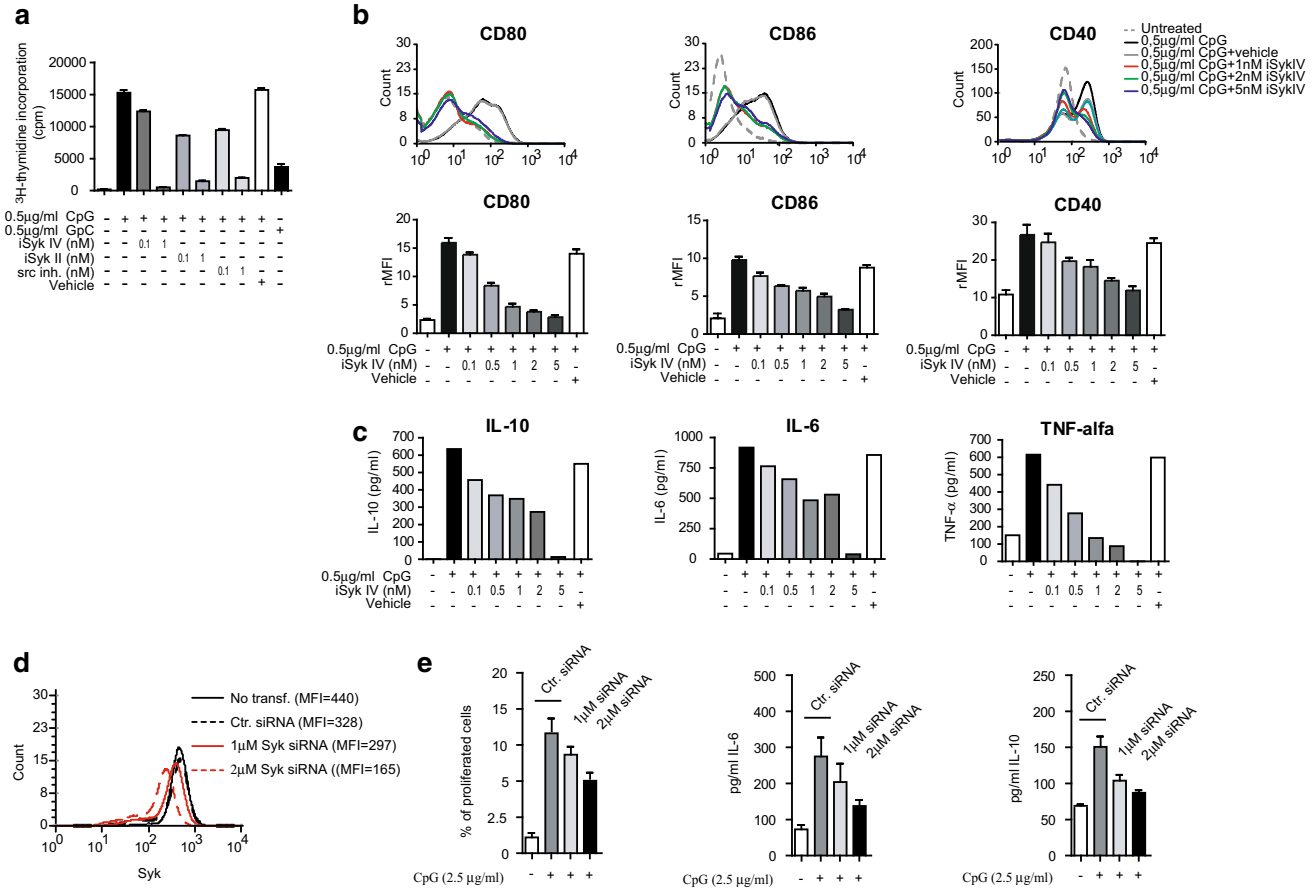

Fig. 2 Syk regulates CpG-driven B cell activation, proliferation and cytokine production (**a**) 2×10^5 resting tonsillar B cells/well were activated with a suboptimal concentration of CpG (0.5 µg/ml) in the presence or absence of Syk or Src inhibitors (iSykIV, iSykII or src inhibitor). As control, cells were cultured in medium or with non-activating GpC ODN. Cells were harvested after pulsing with 1 µCi/well H^3-thymidine for the last 18 h of culture. Results are expressed as normalized mean cpm ± SD of triplicate samples of one representative experiment of 5. **b**, **c** 2×10^5 resting tonsillar B cells/well were activated with 0.5 µg/ml CpG in the presence or absence of different concentrations of Syk inhibitor IV (iSyk IV), as indicated. **b** After 48 h, changes in the expression of CD80, CD86 and CD40 were investigated by flow cytometry. Expression of CD80, CD86 or CD40 is illustrated as flow cytometric histograms as well as by diagrams showing normalized mean relative MFI ± SD of duplicate samples. One representative experiment of 3 is shown. **c** Secretion of IL-10, IL-6 and TNF-α was assessed after the stimulation of resting tonsillar B cells with CpG for 48 h. Results of the Flow Cytomix assay show the amount of secreted cytokines of pooled duplicate samples. One representative experiment of 3 is shown. **d** Expression of Syk in B cells after transfection with 1 µM (*red solid line*) or 2 µM (*red dotted line*) Syk-targeting siRNA. As control, non-transfected (*black solid line*) or 2 µM control siRNA treated (*dotted black line*) B cells were used. Histograms are representative of 3 independent experiments. **e** 1×10^6 B cells/sample transfected with Syk siRNA or control siRNA were left untreated or activated with 0.5 µg/ml CpG. Proliferation of B cells was measured by CFSE and results are illustrated as mean % of proliferated cells ± SD of duplicate samples. IL-6 and IL-10 production were assessed by FlowCytomix assay and data show the amount of secreted cytokines of pooled duplicate samples. Results are representative of 3 independent experiments

found that high-density tonsillar B cells express TLR9 mainly intracellularly (mean relative MFI = 15.63 ± 2.25, $n = 5$); however, low level of TLR9 (with a relative MFI of 2.24 ± 0.35, $n = 5$) can also be detected on the plasma membrane (Fig. 5c). Additionally, we could detect a potentially active, cleaved form of TLR9 in the biotinylated surface protein fraction of B cells (data not shown), suggesting that B cell express a potentially active form of the receptor in the plasma membrane. Indeed, engagement of surface TLR9 by CpG 2006 immobilized on the culture plate initiated a dose-dependent phosphorylation of Syk, Lyn and p38 (Fig. 5d); however, it did not induce B cell proliferation at all, in sharp contrast to the effect of CpG offered to the cells in soluble form (Fig. 5f). Involvement of surface TLR9 in the initiation of Syk-dependent signalling pathways is further strengthened by the increased phosphorylation of the kinase after engagement of surface TLR9 by monoclonal anti-TLR9 Abs (Fig. 5e) or by the

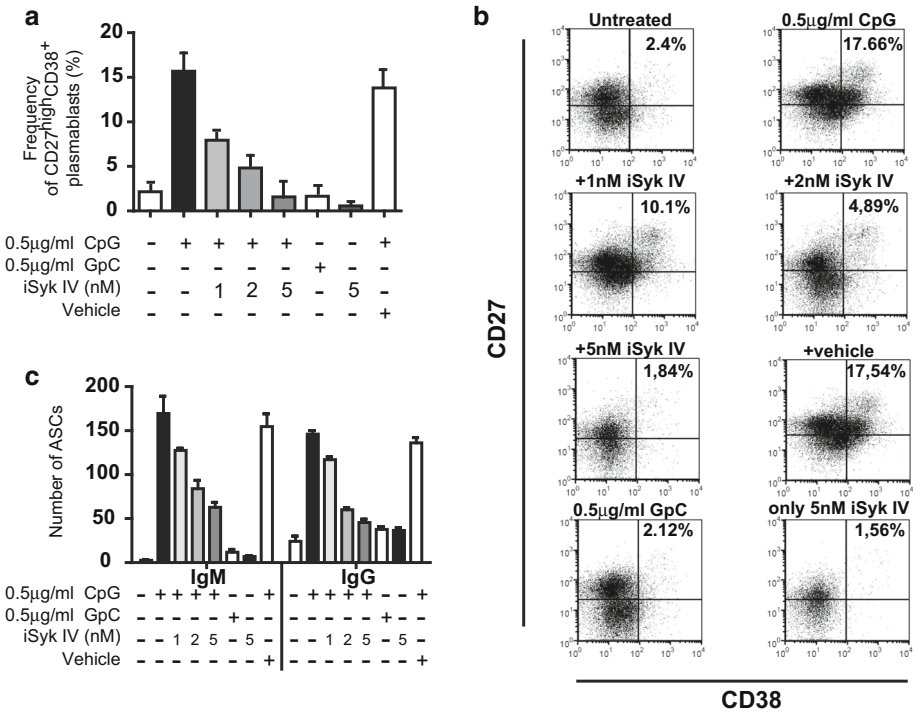

Fig. 3 Syk regulates CpG-induced plasmablast differentiation and Ab production in B cells 2×10^5 resting tonsillar B cells/sample were activated with 0.5 µg/ml CpG in the presence or absence of different concentrations of iSyk IV. **a** The percentage of $CD19^{low}CD27^{high}CD38^{+}$ plasmablasts was determined at day 7 by flow cytometry. Dead cells were excluded by PI staining. Data show mean frequency of $CD19^{low}CD27^{high}CD38^{+}$ cells ± SD of duplicate samples. One representative experiment of 3 is shown. **b** Dot plots of the same samples. **c** The number of IgM and IgG secreting cells was measured by ELISPOT assay at day 7. *Graph* shows normalized mean number of ASCs ± SD of duplicate samples. One representative experiment of 3 is shown

less stable CpG with phophodiester backbone (data not shown).

Inhibition of Syk does not influence the uptake of CpG oligonucleotides

To explore whether the reduced activation, proliferation, cytokine and Ig production are caused by the decreased endocytosis of CpG ODN due to the lack of Syk activity, we studied the uptake of various amounts of fluorescently labeled CpG 2006 by human B cells at different time intervals in the presence or absence of Syk inhibitor IV. As shown in Fig. 6b, uptake of CpG ODN begins already after 5 min of exposure and reaches saturation within 30 min. Investigating both by flow cytometry (Fig. 6a–c) and confocal microscopy (Fig. 6d, e), we found that B cells bind ODN even in the presence of Syk inhibitor IV, suggesting that Syk is not involved in the uptake of CpG ODN by human B cells. Interestingly, however, treatment of the cells with Syk inhibitor IV clearly reduced the colocalization of CpG and its receptor, TLR9. Similarly, the appearance of CpG in TLR9-containing lysosomes were reduced, however, it did not reach statistical significance (Fig. 6d, e). These results suggest that Syk is a prerequisite for optimal delivery of CpG into TLR9-containing endolysosomes.

Syk regulates CpG-driven up-regulation of TLR9 expression in B cells

It has been proposed that signaling through the BCR, CD40 and TLR9 leads to an increased TLR9 expression in B cells [5, 10]. Since we found that Syk blockade strongly inhibits CpG-induced proliferation, cytokine production and differentiation, we assumed that Syk is fundamentally involved in transducing signals which are required to maintain and up-regulate TLR9 expression. Testing this hypothesis by flow cytometric analysis we found that in the presence of 5 nM Syk inhibitor IV, B cells stimulated by CpG for 48 h are unable to up-regulate intracellular TLR9 expression (Fig. 7a). These results were strengthened by Western Blot analysis (Fig. 7b).

Overall, our results indicate that the kinase activity of Syk is a prerequisite for the CpG-induced up-regulation of

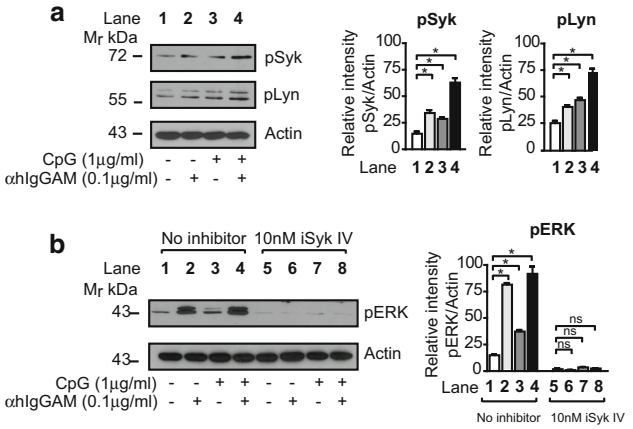

Fig. 4 Inhibition of Syk prevents the BCR and CpG-induced synergistic activation of MAPKs. **a** Phosphorylation level of Syk and Lyn in 2×10^6 high-density B cells/lane that were left untreated (*lane 1*), activated with 0.1 μg/ml $F(ab')_2$ fragment of goat anti-human IgG/A/M (*lane 2*), 1 μg/ml CpG (*lane 3*) or by both stimuli simultaneously (*lane 4*). **b** Phosphorylation level of ERK of 2×10^6 high-density B cells/lane that were left untreated (*lanes 1, 5*), activated with 0.1 μg/ml $F(ab')_2$ fragment of goat anti-human IgG/A/M (*Lanes 2, 6*), 1 μg/ml CpG (*lanes 3, 7*) or by both stimuli simultaneously (*lanes 4, 8*). *Lanes 1–4* show pERK levels in the absence, while *lanes 5–8* in the presence of 10 nM iSyk IV. Immunoblots show results of one representative experiment out of 3. *Graphs* show results of summarized densitometric analysis as mean relative intensity ± SEM of 3 independent experiments (permutation-test, nsP > 0.05; *$P < 0.05$)

TLR9 expression allowing efficient propagation of TLR9-mediated signaling and B cell functions.

Discussion

It is known for long that binding of CpG-containing ODNs to endosomal TLR9 triggers B cell proliferation, inflammatory cytokine and Ab production by a mechanism that is dependent on the adaptor protein MyD88 [3, 4, 12]. In the present study, we demonstrate for the first time that a CpG-induced src kinase-Syk pathway is required for the efficient delivery of CpG into TLR9-containing endosomes as well as for the up-regulation of TLR9 in human B cells. We show here that the Syk-mediated cascade is a prerequisite for optimal induction of TLR9 signaling and a subsequent CpG-driven activation of B cells.

Our results give evidence of a CpG-induced phosphorylation of Syk in resting human tonsillar B cells independent of the BCR. Regarding the CpG-induced activation of Syk, data published earlier are contradictory. It has been shown that the activation of this kinase can be induced by CpG type A DNA in human monocyte-derived dendritic cells independent of TLR9 and by CpG type B ODN 2006 in PBMCs in a TLR9-dependent manner [30, 31]. Furthermore, in two other studies CpG alone did not affect Syk phosphorylation [20, 32]. The discrepancy between these results is most probably caused by differences in the applied ODNs, target cells and antibodies reacting with Syk.

Our major finding is that activation of Syk is indispensable for the most important CpG-driven B cell functions such as Ab and cytokine production. Iwata et al. reported that concurrent activation of B cells through the BCR, CD40 and TLR9 induces robust activation, proliferation, cytokine and Ab production, and all these functions are abrogated by the inhibition of Syk. In this study, the decrease of the B cells' response in the presence of Syk inhibitors was attributed to the lower BCR-induced NFκB phosphorylation and the subsequently reduced up-regulation of TLR9 [32]. Our results exceed these observations, since we found that CpG alone can induce efficient propagation of B cell activation, without the involvement of BCR and CD40. Furthermore, data shown here point to the importance of CpG-driven direct phosphorylation of Syk, since inhibition or silencing of the kinase blocks all kinds of B cell functions. Jabara et al. found that the CpG-induced Syk activation occurs through DOCK8 in PBMCs, resulting in robust proliferation and IgG secretion. In these experiments, DOCK8-induced Syk phosphorylation did not seem to be necessary for the up-regulation of CD86 and cytokine (such as IFN-α) production [30]. These data strengthen our results showing that CpG-induced Syk activation can be initiated not only by intracellular TLR9 in a DOCK8-MyD88-dependent manner, but also via cell surface receptors (including TLR9) which function upstream of DOCK8 and are activated by src-family kinases at a membrane proximal site. Based on this scheme it is conceivable why DOCK8-deficient but not Syk-inhibited cells are able to maintain certain CpG-induced B cell functions such as activation of p38 and secretion of IFNα.

Studying the mechanism of the CpG-induced Syk activation we found that inhibition of CpG internalization did not prevent the phosphorylation of Syk or Lyn, indicating that the activation is initiated by cell surface-bound CpG. Guerrier et al. [23] showed that TLR9 can be expressed on the plasma membrane of human B lymphocytes and engagement of the surface receptor by monoclonal anti-TLR9 Abs results in tyrosine phosphorylation of signaling molecules. Since we also have found that resting tonsillar B cells express TLR9 on the cell surface and inhibition of MyD88 reduces CpG-induced activation of Syk, we suppose that the cell membrane TLR9 is involved in the initiation of Syk phosphorylation. Participation of cell surface TLR9 in CpG-induced Syk phosphorylation was further strengthened by the anti-TLR9 Ab induced

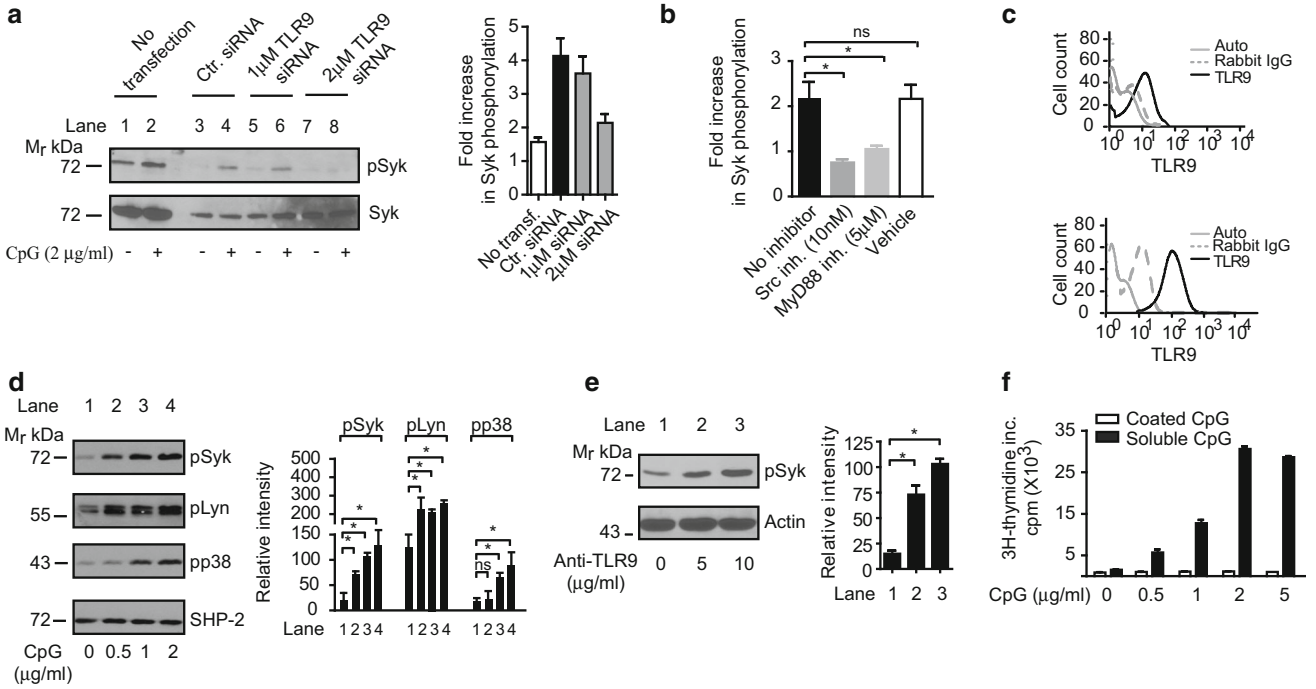

Fig. 5 CpG ODN induces Syk activation in a TLR9-dependent manner in human B cells. **a** Phosphorylation level of Syk in 5×10^5 B cells/sample transfected with TLR9 siRNA or control siRNA that were left untreated (*lanes 1, 3, 5, 7*) or activated with 2 μg/ml CpG (*lanes 2, 4, 6, 8*). Immunoblots show one representative experiment of 2. *Graph* shows summarized densitometric analysis as fold increase in Syk phosphorylation ± SEM of 2 independent experiments (permutation-test, $*P < 0.05$). **b** Activation of Syk was measured in cells that were pre-treated with Src- or MyD88 inhibitors as described in "Materials and methods". *Graph* shows summarized densitometric analysis of Syk phosphorylation as fold increase in Syk phosphorylation ± SEM of 5 independent experiments (permutation-test, nsP > 0.05; $*P < 0.05$). **c** TLR9 expression of high-density tonsillar B cells. *Histograms* show cell surface (*upper panel*) and intracellular (*lower panel*) staining. Data shown are one representative of 5 independent experiments. **d** Phosphorylation level of Syk, Lyn and p38 in 2×10^6 high-density B cells/lane that were left untreated (*lane 1*) or activated by different concentrations of biotinylated CpG coupled to neutravidin coated plates (*lanes 2–4*). Immunoblots show one representative experiment of 3. *Graph* shows summarized densitometric analysis as mean relative intensity ± SEM of 3 independent experiments (permutation-test, nsP > 0.05; $*P < 0.05$). **e** Activation of Syk was assessed after 2×10^6 resting tonsillar B cells/lane were activated with different concentrations of surface-bound anti-TLR9 Ab (clone H-100). Immunoblots show results of one representative experiment of 2. **f** 2×10^5 resting tonsillar B cells/well were activated with different concentrations of soluble (*closed bars*) or surface (neutravidin)-bound biotinylated CpG (*open bars*) for 48 h. Cells were harvested after pulsing with 1 μCi/well H^3-thymidine for the last 16 h of culture. Results are expressed as normalized mean cpm ± SD of triplicate samples of one representative experiment of 3

activation of the kinase and by reduced Syk activation after down-regulation of TLR9 expression by siRNA-mediated gene silencing. All these results support the role of TLR9 in CpG-induced activation of Syk; however,since CpG does not exclusively bind to TLR9, the contribution of additional CpG-binding cell membrane molecules to Syk phosphorylation still cannot be excluded and should be further explored. Based on our data and those published by Jabara et al. [30] we hypothesize that CpG-induced Syk activation is initiated from the cell surface, intersects the intracellular TLR9-MyD88-cascade through Bruton's tyrosine kinase [40] and results in a subsequent, most probably DOCK8-dependent recruitment of Lyn and Syk.

We provide evidence here that a further important consequence of Syk inhibition is the suppression of CpG-induced up-regulation of TLR9 in B cells. The BCR-, CD40- and CpG-driven up-regulation of TLR9 has already been reported earlier; however, its expression has been studied mainly at the mRNA level [5, 10, 36]. Investigating the expression of TLR9 at the protein level, we clearly show for the first time that binding of CpG to human B cells results in up-regulation of its own receptor. This finding points to a positive feed-back loop in which CpG can facilitate its own effects. Thus the blockade of Syk and its downstream signaling molecules leading to enhanced TLR9 expression could inhibit the positive feed-back loop,

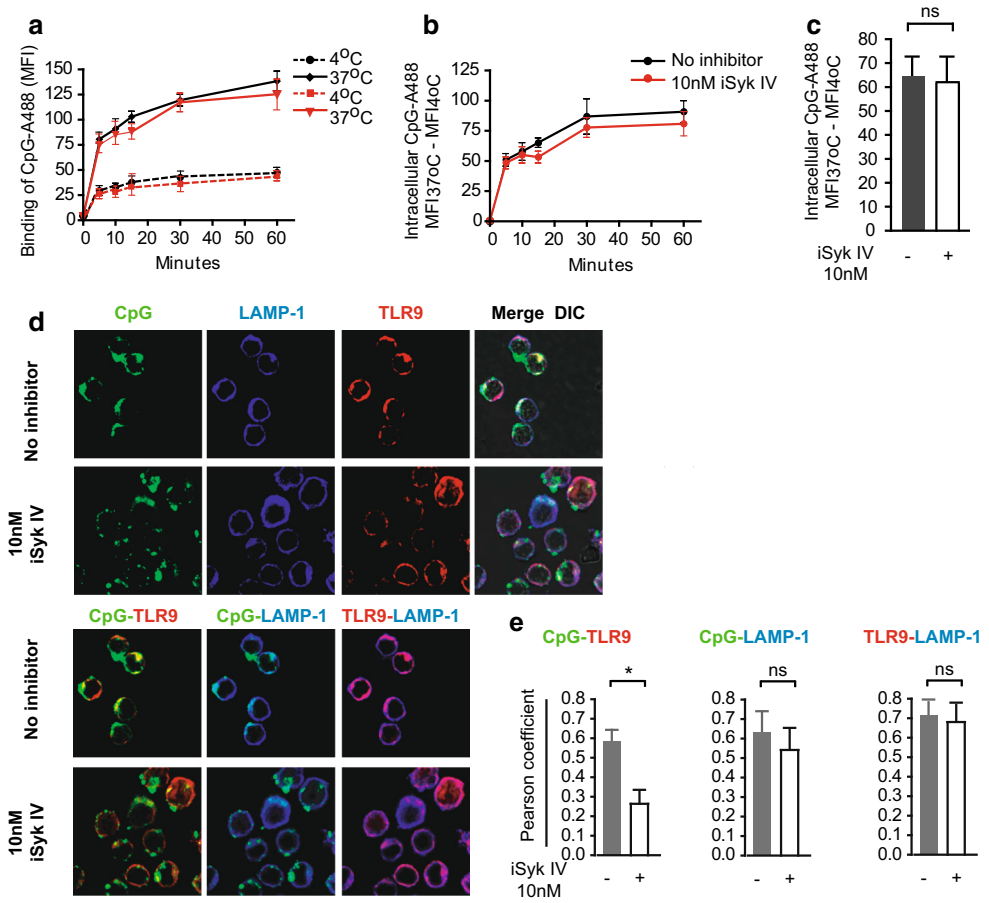

Fig. 6 Syk inhibition does not influence the uptake of CpG ODN. **a**, **b** High-density tonsillar B cells were stained with Alexa Fluor 488-labeled CpG ODN on ice or at 37 °C for the indicated times in the presence (*red lines*) or absence (*black lines*) of 10nM iSykIV. Uptake of the fluorescent ligand was measured by flow cytometry as described in "Materials and methods". Results shown are mean ± SD of one representative experiment out of 3. **c** Uptake of CpG-Alexa Flour 488 after 30 min in the presence or absence of 10nM iSykIV. Results shown are mean ± SEM of 3 independent tests (Permutation-test, nsP > 0.05; *$P < 0.05$). **d**, **e** B cells were stained with Alexa Fluor 488-labeled CpG ODN at 37 °C for 30 min in the presence or absence of 10nM iSykIV. Binding of anti-TLR9 (clone 26C593) and anti-LAMP-1 was visualized using anti-mouse Alexa Flour 647 and anti-rabbit Alexa Flour 555, respectively. Shown are merged DIC and confocal images of CpG-Alexa Flour488 (*green*), LAMP-1-Alexa Flour 555 (*blue*) and TLR9-Alexa Flour 647 (*red*). Cells were analyzed with Olympus FLUOView 500 confocal laser scanning microscope. **d** One representative experiment of 3 is shown. *Scale bars* represent 10 µM. **e** *Graphs* show summarized mean ± SEM of Pearson coefficients of 100 cells for each sample from 3 independent experiments (Student's *t* test, nsP > 0.05; *$P < 0.05$)

thereby blocking the efficient propagation of CpG-induced B cell functions.

The actin cytoskeleton plays an important role in the process of B cell activation by the reorganization of the microtubular-actin network following Ag stimulation. Syk was proven to be essential for optimal actin reorganization in human B cells allowing BCR-induced spreading, Ag internalization and assembly of signalosomes [41–43]. Moreover, Syk-dependent BCR uptake was shown to govern the subcellular location of TLR9 [44]; however, CpG-induced B cell activation was observed without BCR stimulation, as well. Here we show that hindrance of CpG-induced B cell functions in the presence of Syk inhibitor is not due to the impaired uptake of the ODN but is caused most probably by insufficient delivery of CpG into the TLR9-containing endolysosomes. Indeed, CpG and TLR9 colocalization decreases in the presence of Syk inhibitor, suggesting an important role of Syk in the transport of ODNs in human B cells.

In conclusion, we propose the following model for the CpG-induced B cell stimulation. Binding of CpG ODN to TLR9 or other surface receptor(s) results in CpG uptake and initiates Syk phosphorylation commencing from the cell surface. Thereafter activation of Syk induces actin reorganization, which is indispensable for the effective endosomal maturation and trafficking of CpG into TLR9-containing endolysosomes. Here, binding of CpG to its specific receptor induces a MyD88-dependent second wave

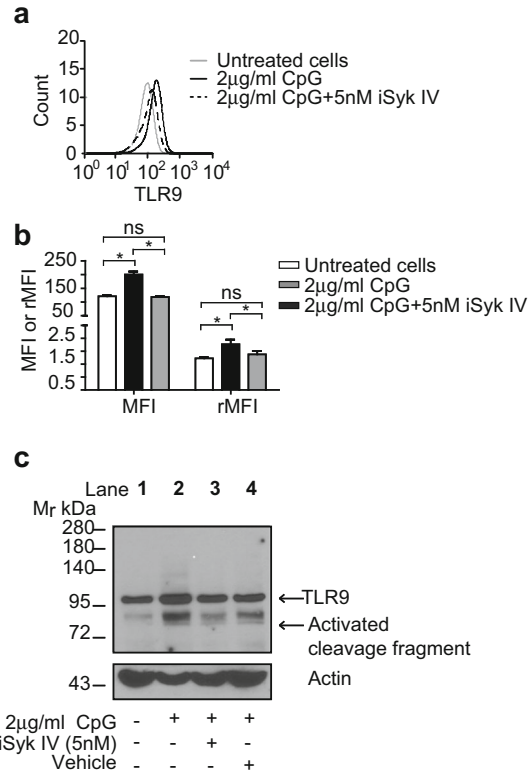

Fig. 7 Syk regulates CpG-driven up-regulation of TLR9 expression in B cells. **a**, **b** Expression of TLR9 after stimulation of B cells with 2 µg/ml CpG in the presence or absence of 5nM iSyk IV for 48 h. **a** Histograms showing intracellular TLR9 expression are as follows: untreated cells (*grey line*), 2 µg/ml CpG treated cells (*black line*), 2 µg/ml CpG and 5nM iSyk IV treated cells (*dashed line*). Results are representative of 3 independent experiments. **b** *Graph* shows summarized mean ± SEM of MFIs or relative MFIs of TLR9 expression of 3 independent experiments (permutation-test, nsP > 0.05; *$P < 0.05$). **c** Extracts of 10^6 high-density tonsillar B cells were separated by SDS–PAGE and immunoblotted with anti-TLR9 Ab. *Arrows* indicate TLR9 of approximately 110 kDa and its activation fragments. Blot shown is representative of 3 independent experiments

phosphorylation of Syk, which causes B cell proliferation, differentiation and TLR9 up-regulation in synergism with the BCR and other cell surface co-receptors.

Since surface TLR9 is able to activate Syk, it might have a role in the delivery of CpG into endosomes and in up-regulation of TLR9 expression. This process may contribute to the enhanced activation of B cells in some inflammatory autoimmune diseases (for example, SLE) in which high level of self-DNA is released from the dying cells. Considering the indispensable role of Syk and TLRs in certain autoimmune diseases [45–47] our data support the potential advantage of Syk inhibitors in the treatment of those B cell-mediated autoimmune diseases where TLR9 might be involved in disease progression.

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Conflict of interest The authors declare no conflict of interest.

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